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ENZYMATIC CONSTITUTION OF *ALSOPHIS* SALIVA AND ITS BIOLOGICAL IMPLICATIONS

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I. Introduction

As long as fifty-eight years ago (Alcock and Rogers, 1902) it was noted that the saliva of certain ostensibly harmless colubrids had toxic properties. Recently, members of the genus *Alsophis* have been suspected to have a toxic saliva. A. S. Rand (MS.) has detailed accounts of *Alsophis portoricensis* feeding behavior and of the effects of bites in man. Neill (1954) relates an instance of toxic effects in himself from an *A. anguilifer* bite.

The purpose of this paper is two fold: 1) to attempt to provide enzymatic confirmation for the tentative conclusion that the colubrid *Alsophis portoricensis* has a truly active salivary secretion (i.e. with venomous properties); and 2) to characterize the activity found in terms of similar enzymes represented in another colubrid and in a crotalid, thus providing a comparative basis for evaluation of the biological implications of this activity.

Natrix sipedon from Florida and *Crotalus atrox* from Texas provided the other preparations. Commercial crystalline *Crotalus* venom was made available by Dr. W. R. Sistrom. The *Natrix* and *Alsophis* preparations were collected by a straightforward method in the laboratory.

II. Collection of Saliva

A small tablet of washed, fine-pore cellulose sponge, previously moistened with distilled water, was inserted in the reptile's mouth with stainless steel forceps. *Alsophis* readily grasped and chewed on the sponge, but with *Natrix* swabbing was necessary.

When relinquished, the sponge was squeezed and rinsed into a tared weighing bottle. The bottle was placed in a chilled desiccator and evacuated until dry over potassium hydroxide at 5°C. This procedure was repeated over the course of several weeks until a quantity deemed sufficient for subsequent tests was collected.

Ten "milkings" coming from two individuals provided 22 milligrams of *Alsophis* preparation over a period of three weeks. Six *Natrix*, each milked once on four occasions equally spaced over the same three weeks, gave a total of 26 milligrams of preparation.

III. Estimation of protein content

Since all known enzymes are proteins, the first step in gaining a fair comparison of activities depends on the total protein content of the saliva. This was estimated for small redissolved duplicate samples of preparation by the Folin-Lowry method (Lowry et al., 1951). Bovine serum albumin was adopted as the reference protein standard. Protein by percent weight for the three preparations is given below (Table 1).

TABLE 1

Preparation	% protein ($\pm 2\%$)
<i>Alsophis</i>	43.0
<i>Crotalus</i>	99.5
<i>Natrix</i>	16.5

Alsophis is here seen to lie between the harmless colubrid and overtly venomous crotalid with respect to potentially active protein content. These results were used to adjust the dissolved preparations used in the later experiments to equivalent protein content.

IV. Spreading factor estimation

The reports of Neill and Rand (above) indicate the presence of a "spreading factor," seen in many natural venoms (Kellaway, 1939). In the case of snake venoms this is probably a complex mixture of proinvasins and hyaluronidase (Zeller, 1948). These enzymes respectively inhibit destruction of the hydrolytic agent of the venom and destroy the mucoid structural material of the cell lattice by a hydrolytic action. The resultant loosening of the matrix favors the passive transport of the venom components of the tissue.

The method followed here has been modified from Fanilli and McClean (1939). It has the disadvantage that specificity is lost and the results are ill-defined. It is well suited to demonstrating low activities however.

Two dilutions of the preparation in question, adjusted to equal protein content, were mixed with equal parts of india ink and 0.9 saline. Of each dilution 0.025 ml. was injected intradermally on the shaved back of an etherized rat. Bovine serum albumin of approximately equal protein content was substituted for the saliva preparations in five otherwise similar control injections. Sites of injection were placed within the shaved area so that each was straddled by two controls. The diameter of each weal was read at one-half hour intervals. Since the progress of the spots was a nearly constant percentage of the original diameter for the first hour and one half, the observations below are for that range (Table 2). Rates fell off sharply after that period; the rate for *Crotalus* fell off sharply after the first half hour.

TABLE 2
mgm preparation
protein injected

	mgm preparation protein injected	% increase in diameter per hour ($\pm 10\%$)
<i>Crotalus</i>	0.125	80
	0.068	55
<i>Natrix</i>	0.125	20
	0.063	0
<i>Alsophis</i>	0.130	50
	0.065	20
Controls 1	0.000	0
2	"	14
3	"	0
4	"	0
5	"	17

Again, according to this index, *Alsophis* shows a definite tendency toward "venomous" activity.

V. Proteinase

Another type of enzyme frequently active in venoms (Zeller, 1948) is proteinase. Work on the genus *Dromicus*, a relative of *Alsophis*, has demonstrated the activity of this enzyme type in extracts of the parotid gland (Donoso-Barros and Cardenas, 1959). Proteinases act hydrolytically to reduce the chain length of proteins and large polypeptides, providing necrotic pre-digestion of the food object and destruction of the tissues. The fortuitous release of histamine from damaged and dissolved

tissue might be expected to enhance any neurotoxic activity in the venom too.

Proteolytic activity was estimated (Northrop et al., 1948) by measuring the residual material giving the Folin-Lowry reaction in an incubation mixture of enzyme and bovine serum albumin after precipitation with 0.1 molar trichloroacetic acid. Quantities of 0.25 mgm protein of preparation and 2.5 mgm of bovine serum albumin per milliliter were incubated in M/15 phosphate buffer at pH 7.2 and 37° C. Saliva preparations alone and albumin alone were also assayed for autogenous proteolysis; the added rates of release in these provided a subtractive correction for the incubation mixtures of preparation and protein.

Proteolysis rate is best taken over the first half hour where first order kinetics appear to obtain. The relative activities are given below, the average of two experiments.

TABLE 3

Preparation	μ gm BSA hydrolyzed/ mgm preparation protein/ hour ($\pm 2\%$)
<i>Crotalus</i>	28.3
<i>Alsophis</i>	12.3
<i>Natrix</i>	0.3

VI. Hemolysis

The lysis of red blood cells is a conspicuous part of the action of snake venoms (Kellaway, 1939). The major part of this effect is due to the action of phospholipase A on tissue phosphatides to produce lysolecithin. This substance renders erythrocytes extremely fragile (Zeller, 1948). The subsequent action of proteolytic enzymes results in the disruption of the cell wall (lysis). Other substances in venoms besides lipases promote lysis without first acting on an intermediate, and it is these which were measured here.

Following Bernheim (1947), aliquots of washed horse erythrocytes were incubated with measured quantities of the various preparations in buffered isotonic saline at 37° C. A blank was run to correct for autolysis in the buffered saline, and one aliquot was lysed by repeated freeze-thawing to give 100% lysis. At the end of the incubation period the cells were centrifuged down and the supernatant liquid was immediately read for absorbance at 579 m μ , an absorption peak of hemoglobin. This quantity when related to that of the completely lysed portion gave a

percentage lysis for the cells. Hemolysis rates are given below as percent lysis per ten micrograms preparation protein per milliliter per hour.

TABLE 4

Preparation	lysis/hour
<i>Crotalus</i>	4.42%
<i>Alsophis</i>	1.20%
<i>Natrix</i>	-0.73%

The negative value for the *Natrix* preparation indicates a lysis rate less than the control, i.e. a protective effect.

VII. Cholinesterase

The typical elapine venom, noted for neurotoxicity, is extremely high in a unique type of cholinesterase (Zeller, 1947). Presumably, since it is present in high concentrations, the curare-like effect of this venom is due to this enzyme's hydrolytic action on acetylcholine. This blocks transmission at neuromuscular junctions, and hence paralyzes the prey. However, inhibitors of this enzyme do not protect animals as fully as might be expected from the effects of cobra venom, including the neurotoxic effects (Zeller, 1948). The situation here is not as clear as might be hoped.

The assay method was essentially that of Hestrin (1949). The preparation was incubated with M/15 phosphate buffer at pH 7.2 and 30° C; acetylcholine concentration was four micromoles per milliliter. From the known protein content of the preparation and the measured disappearance of acetylcholine the activity of the preparation was established. Under the conditions described, accuracy was one tenth of a micromole. This was also the minimum amount of hydrolysis detectable.

TABLE 5

Preparation	Units esterase
<i>Crotalus</i>	0.0
<i>Alsophis</i>	0.2
<i>Natrix</i>	7.4

The unit of activity is conventionally defined as micromoles acetylcholine hydrolyzed per hour per milligram preparation protein under the conditions of the assay. Cholinesterase is normally low or non-detectable in crotaline venoms. The high activity of the *Natrix* saliva is surprising. Subsequent tests showed that the *Natrix* preparation was 50 per cent inhibited

by 10^{-2} molar hexamethonium (1,6-bis hexane trimethylammonium); completely inhibited by 10^{-4} and 90 per cent inhibited by 10^{-6} molar concentrations of neostigmine (prostigmine), respectively. This preparation did not appear to hydrolyze either benzoyl choline or acetyl β -methyl choline. Characteristically, elapine venom cholinesterase does not act on benzoyl choline, but will hydrolyze acetyl β -methyl choline. It was unfortunately impossible to treat the *Alsophis* preparation to this sort of analysis due to its low activity and a waning supply of preparation.

VIII. Discussion

The statement that a particular animal is or is not "venomous" is outwardly a qualitative judgment, but it conceals a quantitative statement. The demonstrated presence of enzymes characteristic of venom in *Alsophis*' saliva constitutes necessary but not sufficient proof for the postulated function of the secretion in subduing prey.

Alsophis' saliva protein content and the relatively greater activity of that protein are superior to those found in the "non-venomous" *Natrix*. Two large teeth surrounded by a fleshy ridge in the rear of the upper maxilla appear to be modified for administration of the saliva. Although these rear teeth are not grooved, the total picture for *Alsophis* indicates a degree of specialization trending in the direction of the adaptations of the truly venomous species. However, these morphological features, like the relative potency of the salivary secretion, are only an indication of the possible function. Critical evidence with regard to the use and thus the significance of the "venom" will probably come from the careful study of feeding behavior.

Animals killed by snake venom decompose at a much more rapid rate than similar individuals who have died peacefully (Zeller, 1948). Vipers, kept from injecting venom by fang removal or ligature of the duct, take from two to three times the normal time to digest a food object of constant size. Apparently the digestive function of venom is a large part of its adaptive significance, especially in an animal unable to mechanically masticate its prey. It may be that at the level of development seen in *Alsophis*, the effect of the saliva in subduing prey is secondary and gratuitous to the digestive function. At higher levels of specialization it is almost impossible to disentangle the two roles. The enzymes of snake venom are most easily interpreted as a digestive complex (Zeller, 1948).

The cholinesterase of *Natrix* saliva, unlike the usual elapid type (Zeller, 1947), will not hydrolyze acetyl β -methyl choline. This is especially interesting in light of the problem of the relationships between the colubrids and elapids (Johnson, 1956). An enzyme study of the analogous secretions of these two groups in a larger range of genera could prove valuable. It would, of course, first be necessary to establish the stability of enzyme type as a character within a genus or genera for which the relationships are relatively clear.

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